Research Note

A *Lotus japonicus* Nodulation System Based on Heterologous Expression of the Fucosyl Transferase NodZ and the Acetyl Transferase NoIL in *Rhizobium leguminosarum*

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Accepted 17 December 1999.

Heterologous expression of NodZ and NoIL proteins in *Rhizobium leguminosarum* bv. *viciae* led to the production of acetyl fucosylated lipo-chitin oligosaccharides (LCOs), indicating that the NoIL protein obtained from *Mesorhizobium loti* functions as an acetyl transferase. We show that the NoIL-dependent acetylation is specific for the fucosyl penta-*N*-acetylglucosamine species. In addition, the NoIL protein caused elevated production of LCOs. Efficient nodulation of *Lotus japonicus* by the NodZ/NoIL-producing strain was demonstrated. Nodulation efficiency was further improved by the addition of the ethylene inhibitor L- α -(2-aminoethoxyvinyl) glycine (AVG).

Additional keywords: acetyl fucose, mass spectrometry, Nod factors.

The induction of root nodules on leguminous plants by rhizobia is mediated by rhizobial Nod factors, which have been identified as lipo-chitin oligosaccharides (LCOs) (Bladergroen and Spaink 1998; van der Drift et al. 1998). Our current studies on the function of Nod factors are being facilitated by the use of the leguminous model plant *Lotus japonicus*, which is very well suited to genetic analyses (Handberg and Stougaard 1992; Quaadvlieg et al. 1998). For the analysis of the activity of Nod factors it is important to have a well-established bacterial expression system for the genes that are involved in their biosynthesis or secretion. These genes, which are collectively called *nod* genes, are designated *nod*, *nol*, or *noe*. The function of *nod* genes can be elegantly demonstrated by a gain of function approach. For example, the activity of the NodZ protein of *Bradyrhizobium japonicus* as a fucosyl transferase was demonstrated by its expression in *Rhizobium leguminosarum* bv. *viciae* and subsequent analysis of the Nod factors produced

(López-Lara et al. 1996). In this paper we use a similar approach to identify the function of the NoIL protein of *Mesorhizobium loti*. An additional goal of these studies was to obtain an efficient heterologous nodulation system for *L. japonicus*, which can also be used for the analysis of factors other than the LCOs involved in infection and nodulation.

In this study, we used *R. leguminosarum* bv. *viciae* RBL5560 expressing the *nodZ* gene (on plasmid pMP2450) and the flavonoid independent transcription activation (FITA) *nodD* gene (on pMP1604) as described previously (López-Lara et al. 1996) (Table 1). Introduction of the *nodZ* and *nodD* FITA genes had been shown to result in extension of the host range of RBL5560 to include tropical leguminous plants, such as siratro. Further analysis of the host range of this strain in this study demonstrated that it is also able to nodulate *L. japonicus* (Fig. 1A). However, nodule frequency and number were rather low compared with those induced by the wild-type *M. loti* strain R7A (Fig. 1A). Since preliminary results indicated that nodulation of *L. japonicus* is strongly inhibited by ethylene (data not shown) we tested the effect of addition of the ethylene production inhibitor L- α -(2-aminoethoxyvinyl) glycine (AVG). Results (Fig. 1A) show that nodulation of *L. japonicus* by both strains was greatly stimulated in the presence of 12.7 nM AVG, and that the wild-type strain remained the more efficient nodulator. We assumed that this failure of RBL5560.pMP2450.pMP1604 to exhibit wild-type nodulation was caused by the fact that wild-type LCOs of *M. loti* bear an acetyl moiety on the fucose residue, in addition to methyl and carbamoyl substituents on the nonreducing residue (López-Lara et al. 1995a), which are not present on the LCOs of the *R. leguminosarum* strains (Spaink et al. 1995a). Our attention in this study was directed at examining the effect of the *nolL* gene on nodulation of *L. japonicus*. It had previously been shown that *nolL* functions in the addition of an acetyl group to the fucose residue (Berck et al. 1999; Corvera et al. 1999; Scott et al. 1996). To analyze the function of the *nolL* gene of *M. loti* in our system it was necessary to reclone the *nodZ* and *nodD* FITA genes into one plasmid in order to introduce an

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additional IncP plasmid harboring the *nolL* gene. Therefore, the *nodZ* gene from pMP2450 was cloned into pMP1604, resulting in an IncW plasmid (pMP 2469) containing both *nodZ* and *nodD* FITA (Table 1). Plasmid pMP2470, an IncP replicon carrying the *nolL* gene, was introduced into strain RBL5560.pMP2469 and tested for nodulation on *L. japonicus*. The onset of nodulation (Fig. 1B) for strain RBL5560.pMP2469.pMP2470 was clearly much earlier than for the strain lacking the *nolL* gene, in both the absence and presence of AVG. The most pronounced effect of the presence of *nolL* was observed in the absence of AVG, when it resulted in an acceleration of the onset of nodulation from 27 to 13 days.

L. japonicus plants infected with RBL5560.pMP2469 (lacking the *nolL* gene) grew poorly and leaves became yellow between 25 and 30 days after inoculation, whereas plants infected with the strain expressing *nolL* (RBL5560.pMP2469.pMP2470) grew as well as plants infected with the wild-type *M. loti* strain R7A and maintained green leaves throughout the duration of the experiment. These observations strongly suggest that nitrogen fixation is taking place in nodules formed in the presence of bacteria expressing *nolL* and that nitrogen fixation is disturbed in nodules formed by plants inoculated with RBL5560. pMP2469.

The pronounced effects of the inclusion of *nolL* and *nodZ* in *R. leguminosarum* bv. *viciae* on nodulation behavior led us to examine the range and structures of the resulting LCOs, initially with thin-layer chromatography (TLC) and subsequently with high-pressure liquid chromatography (HPLC) isolation of LCOs and structural analysis by nano-electrospray hybrid quadrupole time of flight tandem mass spectrometry (ES-Q-ToF-MS). Small-scale cultures of RBL5560.pMP2469 and RBL5560.pMP2469.pMP2470 were grown in the presence of ¹⁴C-labeled *N*-acetyl glucosamine for 18 h at 28°C. The cultures were then extracted with *n*-butanol and the radiolabeled LCOs submitted to a preliminary screening with reversed-phase TLCs (Fig. 2A; Spaink et al. 1992, 1995a). Surprisingly, the presence of the *nolL* gene apparently greatly influenced the total amount of radiolabeled LCOs produced (Fig. 2A, lane 3) as compared with the amounts produced by both the wild-type *R. leguminosarum* bv. *viciae* (Fig. 2A, lane 1) and the strain containing only the *nodZ* gene (Fig. 2A, lane 2). The mobility of the major radiolabeled spot resulting from the

incorporation of *nodZ* is increased (Fig. 2A, compare lanes 1 and 2), as expected.

The culture was scaled up to a total of 1 liter, without the use of a radiolabel, and the total LCOs produced were extracted and purified by reversed-phase HPLC as described (López-Lara et al. 1995b; Fig. 2B). Again, it is clear that the total amount of LCOs produced by the strain harboring *nolL* is much greater than that by the strain lacking *nolL*. It is very unlikely that this difference reflects an effect on the secretion of the LCOs, since an *n*-butanol extraction method developed for the extraction of total LCOs from whole cells was used (Spaink et al. 1994). The fractions obtained from the strain bearing both *nodZ* and *nolL*, corresponding to the material absorbing at 303 nm and eluting in the 30–40% acetonitrile step gradient, were pooled and submitted to ES-Q-ToF-MS analysis. The mass spectrum (data not shown) contains two major pairs of M+K⁺ pseudomolecular ions at *m/z* 1273/1279 and 1518/1524, the latter corresponding to the C18:4 and C18:1 fatty acyl-bearing GlcNAc₅ species having an extra *nolL*-dependent acetyl residue. Very interestingly, the *nolL*-dependent acetyl moiety is not transferred to either the C18:4 or the C18:1 GlcNAc₄ analogues, which are thus observed at *m/z* 1273/1279. There is absolutely no trace of ions corresponding to GlcNAc₄ species bearing an extra acetyl group. In addition, there are low abundance ions at *m/z* 1476/1482 for C18:4 and C18:1 GlcNAc₅ species without the *nolL*-dependent acetyl group. Tandem MS analysis of the ion at *m/z* 1518 for the C18:4 fatty acyl-bearing species (data not shown) yielded B ions at *m/z* 703 (B₂), 906 (B₃), and 1109 (B₄), Y ions at *m/z* 651 (Y₂), 854 (Y₃), and 1057 (Y₄), and, importantly, a β-cleavage ion at *m/z* 1330 arising by loss of an acetylated fucosyl residue, together demonstrating that the additional acetyl moiety is located on the *nodZ*-dependent fucosyl residue. Tandem MS analysis of the ion at *m/z* 1524 (Fig. 3) corresponding to the C18:1-bearing species generated ions at *m/z* 709 (B₂), 912 (B₃), and 1115 (B₄), 651 (Y₂), 854 (Y₃), and 1057 (Y₄), and a β-cleavage ion for the loss of an acetyl fucose at *m/z* 1336. A similar mass spectrometric analysis of the LCOs from the control strain in which the *nolL* gene was not present showed the complete absence of LCOs containing an acetyl-fucose, confirming earlier results of López-Lara et al. (1996).

The structure determination results demonstrate clearly that the *nolL* gene is involved in the transfer of an acetyl to the

Table 1. Strains and plasmids^a

Strains	Description	Source/reference
RBL5560	LPR5045 (<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> cured from its own Sym plasmid) provided with Sym plasmid pJB5JI (= pRL1JI mep::Tn5) derived from <i>R. leguminosarum</i> bv. <i>viciae</i> , Rif ^r	Spaink et al. 1987
R7A	Wild-type <i>Mesorhizobium loti</i> , Rif ^r derived from NZP2238	Sullivan et al. 1995
Plasmids		
pMP604	IncP, Tc ^r , contains <i>nodD</i> FITA	Spaink et al. 1989
pRI40	IncW, sp ^r	Innes et al. 1988
pMP1604	IncW, sp ^r , contains <i>nodD</i> FITA from pMP604 inserted into pRI40	López-Lara et al. 1996
pMP1060	IncP, Tc ^r , contains <i>nodD</i> from <i>R. leguminosarum</i> bv. <i>viciae</i>	Bloemberg et al. 1994
pMP3510	IncP, Tc ^r , contains a polylinker	Spaink et al. 1995b
pMP2450	IncP, Tc ^r , contains <i>nodZ</i> of <i>B. japonicum</i>	López-Lara et al. 1996
pPN383	Contains <i>nolL</i> from NZP2037	Scott et al. 1996
pMP2469	IncW, Sp ^r , Sm ^r , contains <i>nodZ</i> and FITA <i>nodD</i> . <i>nodZ</i> was transferred as a <i>Pst</i> I fragment from pMP2450 into pMP1604	This work
pMP2470	IncP, Tc ^r ; contains <i>NolL</i> from pPN383 inserted into pMP3510 as a 2.4-kb <i>Bam</i> HI fragment	This work

^a Km^r, Sp^r, Tc^r, and Rif^r: kanamycin, spectinomycin, tetracycline, and rifampicin resistance; Inc: plasmid incompatibility group; FITA: flavonoid independent transcription activation.

fucose residue. These results are consistent with those obtained previously (Corvera et al. 1999; Berck et al. 1999) for the *nolL* genes of *R. etli* and *Rhizobium* sp. strain NGR234, respectively. Considering the homology of the predicted Noll protein sequence with that of other proteins assumed to be acetyltransferases (Scott et al. 1996), this function for Noll is not surprising. What is remarkable, however, is that the substrate specificity of the Noll protein appears to be restricted in the same way as that of the NodX protein, both transferring an acetyl group only to the GlcNAc₅ species (Firmin et al. 1993). Since the two predicted protein sequences have only 25% amino acid identity, this striking conservation of acceptor specificity could not have been predicted. Even more unexpected is the observation that the Noll protein greatly increases the quantity of LCOs produced. The introduction of

such a new activity might have been expected to decrease rather than to increase the activity of the other biosynthetic enzymes involved in LCO production. Currently, we have no obvious explanation for this phenomenon. The acetyl transferase NodL has also been shown to increase the production of Nod factors (Spaink et al. 1992), the molecular basis for which is also not understood. The increased LCO production is very convenient for obtaining large quantities of suitably modified LCOs for both biological and structural studies. However, it makes it difficult to conclude whether the improved nodulation characteristics conferred by *nolL* are due to its transacetylation activity or purely to the larger quantities of LCOs produced.

In addition to the advantages of a heterologous expression system for demonstrating the biochemical function of a pro-

Fig. 1. Effects of expression of the *nodZ* and *nolL* genes on nodulation of *Lotus japonicus* plants infected with *Rhizobium leguminosarum* bv. *viciae*. **A** and **B**, Percentage of plants with nodules. **C** and **D**, Average number of nodules per nodulated plant, with the strains listed below for the two sets of experiments (**A-C** and **B-D**). Nodulation characteristics were compared with those of the wild-type *Mesorhizobium loti* strain R7A. Seeds were surface sterilized with 1 M H₂SO₄ for 2 min and, after being washed with water, incubated in bleach for 10 min, and washed again with water overnight, vernalized for 2 days at 4°C on agar plates. Germination was performed overnight at 28°C. Plants were grown in petri dishes containing Jensen medium solidified with 1.2% Daishin agar in which the roots were shielded from light. Incubation was at 20°C in a growth room with an 8 h dark, 16 h light cycle. When added to the plates, the ethylene inhibitor L- α -(2-aminoethoxyvinyl) glycine (AVG) concentration was 12.7 nM. Results shown are for one representative experiment out of three performed with 50 plants for each inoculation with RBL5560. *M. loti* R7A inoculations were performed with 10 plants. Indicated values are averages; error bars indicate standard errors (**C** and **D** only). Circles (●): RBL5560.pMP1604.pMP2450; squares (◻): *M. loti* R7A; diamonds (◆): RBL5560.pMP2469.pMP2470; triangles (▲): RBL5560.pMP2469. Continuous and dashed lines represent presence and absence of AVG, respectively.

tein, as demonstrated here, there are also several obvious advantages for biological studies. These include the absence of a genetic background that could include genes having the same biological function as the gene under study. A good example is that of the *nodO* gene of *R. leguminosarum* bv. *viciae*, encoding a secreted protein that is able to complement defects in genes responsible for modifications of the endogenous LCOs

(Downie and Surin 1990). Our test system can be used to analyze the effect on nodulation of *L. japonicus* of well-characterized mutations in *R. leguminosarum* genes, such as, for instance, those involved in exopolysaccharide (EPS) biosynthesis or avirulence (Pollock et al. 1998; Roest et al. 1997). The availability of an efficient, heterologous nodulation system for *L. japonicus* will enable our further studies of bacterial

Fig. 2. Purification of lipo-chitin oligosaccharides (LCOs). **A**, Thin-layer chromatography (TLC) analysis of LCOs. Lane 1: RBL5560. Lane 2: RBL5560.pMP2469. Lane 3: RBL5560.pMP2469.pMP2470. **B** and **C**, High-pressure liquid chromatography (HPLC) profiles of *n*-butanol extracts from (B) RBL5560.pMP2469 and (C) RBL5560.pMP2469.pMP2470 cultures. Amount of sample loaded was equivalent to that isolated from 100 ml of culture. Purification protocol is described in López-Lara et al. (1995a). Isocratic elution with 30% acetonitrile (20 min) was followed by 30 min of isocratic elution with 40% acetonitrile. HPLC elution was performed at a flow rate of 1 ml/min. There are differences in scale between the two chromatograms.

Fig. 3. Tandem mass spectrum and fragmentation diagram obtained from the $M+K^+$ pseudomolecular ion for V(18:4,AcFuc) at m/z 1524.

host-specific genes required for efficient infection and nitrogen fixation in *L. japonicus* with a similar gain of function approach.

ACKNOWLEDGMENTS

It is a pleasure to thank Peter Hock for his skillful assistance in preparing the figures. J. T.-O. gratefully acknowledges financial support (to S. J. Gaskell and R. J. Beynon) from the Higher Education Funding Council for England, with additional support from Smith Kline Beecham, UMIST, University of Wales College of Medicine and Chugai Pharmaceuticals, for the purchase of the Q-ToF.

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